Applied and Environmental Microbiology, Apr. 2008, p. 2537–2539 Vol. 74, No. 8
0099-2240/08/$08.00+0 doi:10.1128/AEM.02288-07

Removal of Free Extracellular DNA from Environmental Samples by Ethidium Monoazide and Propidium Monoazide▽

Andreas O. Wagner,* Cornelia Malin, Brigitte A. Knapp, and Paul Illmer

University of Innsbruck, Institute of Microbiology, Technikerstrasse 25, A-6020 Innsbruck, Austria

Received 8 October 2007/Accepted 13 February 2008

Recently, new DNA extraction techniques (using ethidium monoazide and propidium monoazide) have been developed to discriminate between alive and dead bacterial cells. Nevertheless, for complex environmental samples, no data are available yet. In the present study, these new methods were applied to anaerobic-fermentor sludge and the results were compared to a conventional microbiological approach.

For pathogen risk assessment and hygienic safety control in anaerobic digesters, various culture-based microbiological methods are in use. However, with the application of classical methods, a number of problems arise: long cultivation times for some microorganisms, the complexity of anaerobic cultivation, and time-consuming lab work (enrichment of selected organisms, selective cultivation, and subsequent systematic differentiation). Alternatively, molecular tools could be used, but fast and easy methods, such as PCR amplification after conventional DNA extraction, do not always guarantee the amplification of viable cells' DNA only (6), which might result in false-positive data (9). On the other hand, RNA-based approaches, which would target the active part of a microbial community, thus enabling discrimination between living and dead cells, encounter problems with the high RNA decay rates after the loss of cell viability (1) and are also expensive and laborious.

A new DNA extraction technique including an additional step to remove free, extracellular DNA and DNA of dead bacterial cells by using light-activated ethidium monoazide (EMA) or propidium monoazide (PMA) was described previously, noting the possibility of a selective suppression of DNA detection in dead cells (10, 11, 15). To our knowledge, these extraction procedures were tested successfully with a simple matrix (12), whereas an evaluation of environmental matrices, such as the sludge of an anaerobic digestion plant, has not yet been performed.

The aim of this work was to test the suitability of EMA and PMA for the extraction of free DNA and DNA originating from dead cells in an environmental matrix. The extracted DNA was subsequently amplified via real-time PCR (quantitative PCR [qPCR]) using specific primers for selected pathogenic microorganisms (*Clostridium perfringens*, *Listeria monocytogenes*, and *Salmonella enterica*), and the results were compared to classical cultivation-based agar plating data.

The following organisms, selected after an Austrian standard guideline (14), and an anaerobic spore-forming microorganism, were used after microscopic verification and selective plate counting: *Clostridium perfringens* (DSM 11780; German Collection of Microorganisms and Cell Cultures, http://www.dsmz.de), *Listeria monocytogenes* (DSM 15675), and *Salmonella enterica* subsp. *enterica* serovar Senftenberg (DSM 10062). Pure cultures of *L. monocytogenes* and *C. perfringens* were grown in DSM medium 92 (30.0 g Trypticase soy broth, 3.0 g yeast extract, 1,000 ml distilled water, pH 7.0), and pure cultures of *S. enterica* were grown in DSM medium 220 (15.0 g peptone from casein, 5.0 g peptone from soy meal, 5.0 g NaCl, 1,000 ml distilled water, pH 7.3) for 24 h at 37°C. *L. monocytogenes* and *S. enterica* were cultured aerobically and *C. perfringens* anaerobically in flasks closed with rubber septa. The headspace of culture flasks of *C. perfringens* was exchanged with gas consisting of 30% CO_2 and 70% N_2. The following selective media and corresponding supplement solutions, respectively, were used for plate counting: Oxoid CM0587 and SR0093E for *C. perfringens* agar base TSC/SFP, Merck 1.11755 and 1.12122 for *L. monocytogenes* PALCAM-*Listeria*-Selektivagar, and Oxoid CM0099 for *S. enterica-Salmonella-Shigella* agar.

Fermentor sludge derived from an anaerobic, thermophilic (50°C) biowaste treatment plant in Roppen, Austria, was diluted in distilled water (1:5) as the use of diluted fermentor sludge (DFS) was necessary for better handling. Samples were taken at the outlet sampling port of the fermentor, so the sludge had already been treated in the fermentor for about 14 days. A characterization of the biogas plant and some basic properties of the sludge are given elsewhere (8).

Cell counts of pure cultures of *C. perfringens*, *L. monocytogenes*, and *S. enterica* for the initial inoculum of the DFS were obtained by microscopic counting via a Thoma chamber. Cells were added to DFS to reach a final concentration of 4×10^6 cells ml^{-1} for each organism and exposed to a heat treatment of 50°C, a temperature commonly used for thermophilic anaerobic digestion. Samples were taken in triplicate after 0, 3, 7, and 24 h, and plate counting was done on selective agars from the appropriate dilutions.

For all DNA extractions, 250 μl of sample, corresponding to 1×10^6 cells per microorganism, was processed using the PowerSoil DNA extraction kit (Mo Bio Lab, Inc.). To enhance cell lysis, all samples were subjected to two cycles of freezing (1 h at −80°C) and thawing (30 min at 37°C) after the bead-beating step.

A total of four different DNA extraction approaches was

* Corresponding author. Mailing address: Institute of Microbiology, University of Innsbruck, Technikerstr. 25, A-6020 Innsbruck, Austria. Phone: 43 512 507 6006. Fax: 43 512 507 2928. E-mail: Andreas.Wagner@uibk.ac.at.

▽ Published ahead of print on 22 February 2008.

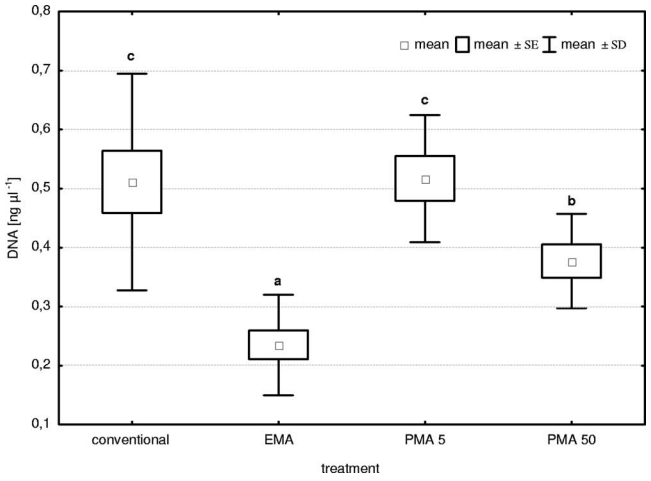

FIG. 1. Amounts of total extracted DNA (PicoGreen measurement) as a result of different extraction procedures. Significant differences ($P < 0.05$) are symbolized by different letters. For an explanation, refer to the text.

performed: (i) DNA extraction after a conventional procedure in accordance with the manufacturer's protocol (see above), (ii) DNA extraction with an additional EMA application step in accordance with the protocol described in reference 10, (iii) DNA extraction with an additional 5 μM PMA application step, and (iv) DNA extraction with an additional 50 μM PMA application step (11). The two different PMA concentrations were chosen since different references can be found in the literature (11, 12). The extracted DNA was quantified using PicoGreen double-stranded DNA quantification reagent (Anthos-Zenyth multimode detector; Invitrogen, Carlsbad, CA) by following the procedure described in reference 7 and used as the template for subsequent organism-specific real-time PCR (qPCR).

Species-specific primer sets were used for *C. perfringens*, *L. monocytogenes*, and *S. enterica*. Primers CP1 and CP2 were used for the selective amplification of *C. perfringens* targeting a 279-bp-specific region of the 16S rRNA gene (3). The conditions were as follows: initial denaturation at 95°C for 5 min and 40 cycles of 15 s at 95°C, 15 s at 60°C, and 60 s at 72°C (modified from a protocol described in reference 3). For *S. enterica*, primers Styinva-left and Styinva-right were used under the following conditions: 35 cycles of 95°C for 15 s and 55°C for 60 s (modified from a protocol described in reference 5). *L. monocytogenes* was amplified with primers described by Nogva et al. (13). The modified amplification protocol included 35 cycles of 95°C for 20 s and 60°C for 60 s.

qPCR was performed on a Rotor-Gene RG 6000 system (Corbett Research) by using a QuantiMix Easy SYG kit (Biotools B&M Labs). The reaction mixture consisted of 7 μl QuantiMix, 1 μl (3.33 μM) of each primer, and 1 μl of the undiluted DNA template. A DNA extract of the untreated samples served as standard DNA for the quantification of microbial cells. qPCR was performed in triplicate. The significance of differences was investigated by analysis of covariance, with exposure time being the continuous covariable.

In Fig. 1, the amounts of the total extracted DNA (PicoGreen measurement) are shown. Conventional DNA extraction resulted in the highest DNA concentrations. By a covariance analysis, significant differences ($P < 0.05$) between EMA, 50 μM PMA, and 5 μM PMA and conventional treatment were established, whereas no significant impact of the duration of heat treatment on total DNA extraction could be detected. The additional EMA treatment step during DNA extraction showed the highest DNA-binding capacity, followed by 50 μM PMA. No significant difference regarding DNA extraction efficiency was found between 5 μM PMA and conventional treatment. However, the results of total DNA measurement have to be interpreted carefully since interactions of EMA or PMA with fluorescent dyes, such as ethidium bromide and PicoGreen, are possible (4).

TABLE 1. Number of surviving cells of *S. enterica*, *L. monocytogenes*, and *C. perfringens* after heat treatment in DFS

Length of heat treatment (h)	Mean no. of surviving cells (SD)[a]		
	S. enterica	*L. monocytogenes*	*C. perfringens*
0	1.4×10^5 (3.86×10^4)	2.4×10^6 (7.26×10^5)	2.1×10^6 (4.09×10^5)
3	7.8×10^0 (1.1×10^1)	8.0×10^3 (4.44×10^3)	7.1×10^2 (2.67×10^2)
7	0	0	5.6×10^1 (8.82×10^0)
24	0	0	6.7×10^0 (7.07×10^{-1})

[a] Data were obtained by plate counting.

The temperature exposure resulted in a distinct decrease in number of culturable cells on selective medium for all test organisms (Table 1). After 7 h of heat treatment, no cells of *L. monocytogenes* or *S. enterica* could be recultivated, whereas for *C. perfringens*, a mean of 7 CFU ml^{-1} (±0.7) was still found after 24 h. Melt analysis of the amplified DNA via qPCR resulted in unique melting temperatures for each microorganism, pointing to a specific amplification of the selected template DNA fragment. In Fig. 2 the differences between the classical and molecular biological approaches are depicted for *S. enterica* and the DNA-binding capacities of EMA and PMA in the different DNA extraction procedures and their consequences on qPCR are shown. While conventionally extracted

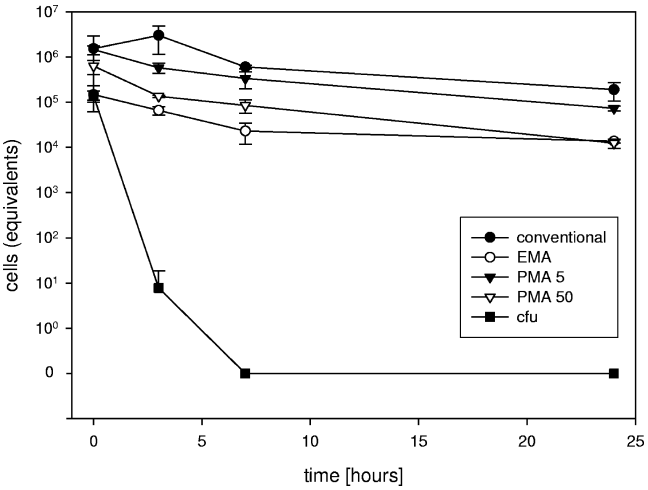

FIG. 2. Survival of heat-treated cells of *S. enterica* in DFS. The results of a comparison of different DNA extraction procedures (conventional, EMA, 5 μM PMA, and 50 μM PMA) with subsequent qPCR and a classical microbiological approach (CFU) are shown.

samples showed the fastest increase of fluorescence in qPCR, 5 μM PMA, 50 μM PMA, and EMA extraction results showed slower increases, a tendency that was observed for entire nucleic acids (Fig. 1) and for the other organisms as well. Analysis of covariance established a significant difference between conventional and EMA treatments ($P < 0.01$), whereas the differences in qPCR results for PMA treatments were not significant. For EMA and 50 μM PMA extractions, a mean reduction of approximately 93% was detected after 24 h of heat exposure compared to that for the conventional extraction, which confirms the general ability of these chemicals to selectively remove the DNA of nonvital cells. However, depending on the specific problem and the initial cell densities, the remaining DNA may still be of relevance. Differences between EMA and PMA and problems such as the targeting of DNA from nonvital cells by EMA under certain conditions are discussed by Nocker et al. (11).

Despite the removal of free and nonvital cells' DNA by the different extraction variations, as assumed by lower DNA yields for EMA and PMA extractions, the most striking difference was found between results from the plate counting and the molecular biological approach.

Although culture-based methods in general and the use of selective medium are known to underestimate the real viable population by a too-weak recultivation of damaged, senescent, or dormant cells, the huge discrepancy between data from the plate count and molecular approaches cannot be explained only by the use of selective medium (2). Therefore, it is likely that, despite the application of EMA and PMA, DNA extraction still leads to false-positive results. This might be explained by the complex nature of DFS, in which interactions between DFS particles and DNA or EMA/PMA seem likely. Moreover, the dark black appearance of the DFS might inhibit the cross-linking step when the chemicals should be light activated since the radiation probably cannot penetrate the liquid.

Although a remarkable reduction of extracted bacterial DNA was observed due to the use of the described EMA- and PMA-based DNA extraction procedures, these methods do not seem to be effective enough to remove the DNA of all nonvital cells in matrices such as DFS, so an overestimation of bacterial viability in DFS by qPCR is still likely. These false-positive results may be especially relevant with high initial cell densities. Hence, classical microbiological methods should be additionally performed, especially in environmental studies, as long as false-positive results from the application of a molecular approach are likely.

The study was supported by the Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management (BMLFUW) and the counties of Tyrol and Vorarlberg (project no. 1405).

REFERENCES

1. **Belasco, J.** 1993. mRNA degradation in prokaryotic cells: an overview, p. 3–12. *In* J. Belasco and G. Brawerman (ed.), Control of messenger RNA stability. Academic Press, Inc., San Diego, CA.
2. **Busch, S. V., and C. W. Donnelly.** 1992. Development of a repair-enrichment broth for resuscitation of heat-injured *Listeria monocytogenes* and *Listeria innocua*. Appl. Environ. Microbiol. **58:**14–20.
3. **Deplancke, B., O. Vidal, D. Ganessunker, S. M. Donovan, R. I. Mackie, and H. R. Gaskins.** 2002. Selective growth of mucolytic bacteria including *Clostridium perfringens* in a neonatal piglet model of total parenteral nutrition. Am. J. Clin. Nutr. **76:**1117–1125.
4. **Hein, I., G. Flekna, and M. Wagner.** 2006. Possible errors in the interpretation of ethidium bromide and PicoGreen DNA staining results from ethidium monoazide-treated DNA. Appl. Environ. Microbiol. **72:**6860–6862.
5. **Hoorfar, J., P. Ahrens, and P. Rådström.** 2000. Automated 5′ nuclease PCR assay for identification of *Salmonella enterica*. J. Clin. Microbiol. **38:**3429–3435.
6. **Josephson, K. L., C. P. Gerba, and I. L. Pepper.** 1993. Polymerase chain reaction detection of nonviable bacterial pathogens. Appl. Environ. Microbiol. **59:**3513–3515.
7. **Juen, A., and M. Traugott.** 2005. Detecting predation and scavenging by DNA gut-content analysis: a case study using a soil insect predator-prey system. Oecologia **142:**344–352.
8. **Malin, C., and P. Illmer.** 2007. Ability of DNA content and DGGE analysis to reflect the performance condition of an anaerobic biowaste fermenter. Microbiol. Res. [Epub ahead of print.] doi:10.1016/j.micres.2007.07.004.
9. **Masters, C. I., J. A. Shallcross, and B. M. Mackey.** 1994. Effect of stress treatments on the detection of *Listeria monocytogenes* and enterotoxigenic *Escherichia coli* by polymerase chain reaction. J. Appl. Bacteriol. **77:**73–79.
10. **Nocker, A., and A. K. Camper.** 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoacide. Appl. Environ. Microbiol. **72:**1997–2004.
11. **Nocker, A., C. Y. Cheung, and A. K. Camper.** 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J. Microbiol. Methods **67:**310–320.
12. **Nocker, A., K. E. Sossa, and A. K. Camper.** 2007. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. J. Microbiol. Methods **70:**252–260.
13. **Nogva, H. K., K. Rudi, K. Naterstad, A. Holck, and L. Lillehaug.** 2000. Application of 59-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. Appl. Environ. Microbiol. **66:**4266–4271.
14. **ÖNORM S2204.** 2007. Kompost-hygieneanforderungen, p. 1–16. Österreichisches Normungsinstitut, Vienna, Austria.
15. **Rudi, K., B. Moen, M. Dromtorp, and A. L. Holck.** 2005. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl. Environ. Microbiol. **71:**1018–1024.